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Stationary phase with specific surface properties for the separation of estradiol diastereoisomers

B. Buszewski*, M. Jezierska-Świtała, S. Kowalska

Department of Environmental Chemistry and Ecoanalytics, Faculty of Chemistry, Nicolaus Copernicus University, 7 Gagarin Street, Pl-87-100 Toruń, Poland

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Abstract

The aim of this study was to develop a procedure that enabled the separation of estradiol diastereoisomers. For this purpose a series of stationary phases with different surface properties has been utilized. Two of them contain various interaction sites, such as: cholesterol, *n*-acylamide, amine and silanols localised in the organic layer bonded to the surface of silica gel (SG-CHOL and SG-CHOL/AP). The other one contains mainly alkylamide ligands and also residual aminopropyl and silanol groups (SG-AP), as well as the last one consisting of hydrocarbonaceous material (SG-C_{18}). In order to select the best type of stationary phase for this analysis, after chromatographic separation of 17-α-estradiol and 17-β-estradiol, selectivity and resolution of the analytes were compared. The best separation of hormones was obtained for SG-CHOL packing, as a consequence of the structure and the properties of this stationary phase. To better understand the retention mechanism and the properties of the stationary phases, used in the separation of steroid compounds, the functional group contributions (τ) were compared with Hansch substituent constants (π).

Keywords: Stationary phases, LC; Estradiol; Steroids

1. Introduction

At the present time high-performance liquid chromatography (HPLC) is one of the most widely used analytical methods. This technique permits the separation of different multicomponent mixtures, and also the identification of compounds and their quantitative analysis. This is connected to the evolution which took place in the preparation of new generations of stationary phases. Many of these materials are related to natural, biological systems and so give the possibility of simulating processes going on at the phase borders (e.g. liquid–solid, liquid–liquid, liquid–gas, etc.), biological barriers (brain–blood) and environments (external and internal environment of the cell). Therefore, the selection of an appropriate stationary phase for a given chromatographic analysis is often the most important step, which determines the quality of the final results. One of the factors playing a predominant role in the separation process is the interaction between solute, stationary phase and mobile phase [1–3].

*Corresponding author. Tel.: +48-56-6114308; fax: +48-56-6114837.
E-mail address: bbusz@chem.uni.torun.pl (B. Buszewski).

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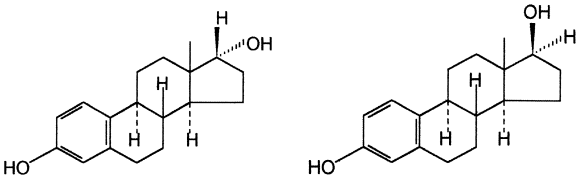
Different stationary phases with diverse properties are recommended for the HPLC analysis of biologically active compounds. Generally, according to Unger's nomenclature [4], they may be focused into several groups, such as packings with hydrophobic character [5–8] and also with hydrophilic character [8–10]. Packings of *monomer* and *polymer* type [6,11] with low and high surface coverage of the support (including with controlled coverage density) also belong here [12]. Finally, in this group are packings with specific surface properties such as: shielded [13–15], chiral [16,17], peptide [18], liquid crystalline [19] and/or immobilized artificial membrane (IAM) stationary phases [20], etc. These last materials, designed by immobilizing phospholipids on a silica surface [20] are especially of interest in the field of researchers–chromatographers and often are applied to specific determinations and modeling of a new generation of drugs. They are also used with success in biomedical applications in the search for analogies to natural systems, especially biological membrane–cell interior systems (cell biology, molecular biology) [21–23].

One of the components of the bilayer biological membrane is cholesterol. This compound can perform the role of an artificial membrane after chemical immobilization on the silica surface [24]. This new generation stationary phase with specific properties seems to fulfil the expectations of chromatographers in the steroid separation represented by estradiol diastereoisomers [24]. Optical isomers often possess widely different biological activity and methods for their isolation are very important. Estradiols are the most active among all the estrogens in the human body, responsible for stimulating growth of the female reproductive system. Sex drive, thyroid function, and skin elasticity are also affected by this hormone. 17-α-Estradiol is the most active of all well-known female sexual hormones, while the isomer 17-β-estradiol is almost not active (Fig. 1).

In the current work, a series of chemically bonded stationary phases with specific structural properties has been synthesized. Two of them contain cholesterol ligands bonded to silica (SG-CHOL, SG-CHOL/AP), one is an alkylamide packing (SG-AP) and the last of them is an octadecyl column (SG-C_{18}) acting as a reference material. For a better understanding of the mechanism of separation on newly synthesized stationary phases with special properties, and in order to interpret the results obtained in this study, the functional group contributions (τ) were compared with Hansch substituent constants (π) [25].



17-α-estradiol 17-β-estradiol

Fig. 1. Structure of estradiol diastereoisomers.

2. Experimental

2.1. Materials and reagents

All stationary phases were synthesized using the highest quality silica gel, Kromasil-100-5-SIL AT0153 (Akzo Nobel, Bohus, Sweden). Physicochemical characteristics of the bare silica adsorbent are presented in Table 1. For the chemical modification of the silica surface, the following reagents were used: octadecyltrichlorosilane purchased from Petrarch System (Levittown, PA, USA); γ-aminopropyltriethoxysilane and triethylamine (Fluka, Buchs, Switzerland); palmitoyl chloride (Merck, Darmstadt, Germany). Cholesteryl chloroformate 98%, lauric acid chloride and 1-octadecene were purchased from Sigma–Aldrich (Gillingham, Dorset, UK). Organic solvents were of HPLC grade (T.J. Baker, Deventer, The Netherlands). Water was taken from a Milli-Q RG system (Millipore Intertech, Bedford, MA, USA).

2.2. Bonded phase synthesis and column packing procedure

Silica support surface modification was carried out in a glass reactor described in Refs. [13,24]. The

Table 1
Physicochemical structure of pure Kromasil 100-5-SIL, AT0153

Characteristics	Abbreviation	Unit	Value
Mean particle size	d_p	μm	5
Particle shape	–	–	Spherical
Specific surface area	S_{BET}	$m^2\ g^{-1}$	310
Pore volume	V_p	$cm^3\ g^{-1}$	0.82
Mean pore diameter	D	nm	10
Concentration of OH groups	α_{OH}	$\mu mol\ m^{-2}$	7.1
Trace amounts of metals	C_M	ppm	
	$C_{M,\ Na}$		28
	$C_{M,\ Fe,\ Al}$		<10

reaction mechanism and the conditions for alkylamide phase synthesis (SG-AP) were the subject of Refs. [13,26,27]. Octadecyl material (SG-C_{18}) was prepared in non-solvent conditions, described previously [26,27]. The procedure for the preparation of SG-CHOL and SG-CHOL/AP phases was delineated in Ref. [24]. Both these packing materials were synthesized in two steps. The first step was described in detail earlier [26] and the second stage was the subject of Ref. [13]. The prepared stationary phases were packed into 100 mm×4.6 mm I.D. stainless-steel tubes purchased from Supelco (Bellefonte, PA, USA). The modified silica was shaken in an ultrasonic bath for 5 min with 15 ml of 2-propanol. All HPLC columns were packed using a laboratory-made apparatus equipped with a Haskel packing pump (Burbank, CA, USA) under a constant pressure of 50 MPa. Methanol was used as a driving solvent.

2.3. Apparatus

A 1050 HP apparatus (Hewlet Packard, Waldbronn, Germany) consisting of a gradient pump, a diode array detector, autosampler and the Vectra QS/HP computer with ChemStation-2 for data collection and instrument control, were selected for chromatographic measurements.

For computer modelling a HyperChem package with the ChemPlus extension (HyberCube, Waterloo, Canada), Statgraphics package (Manugistics, Rockville, MD, USA), DataFi (Oakdale Engineering, Oakdale, PA, USA) were used.

3. Results and discussion

3.1. Surface characterization

The schematic structures of the synthesised stationary phases, used in this study, are illustrated in Fig. 2.

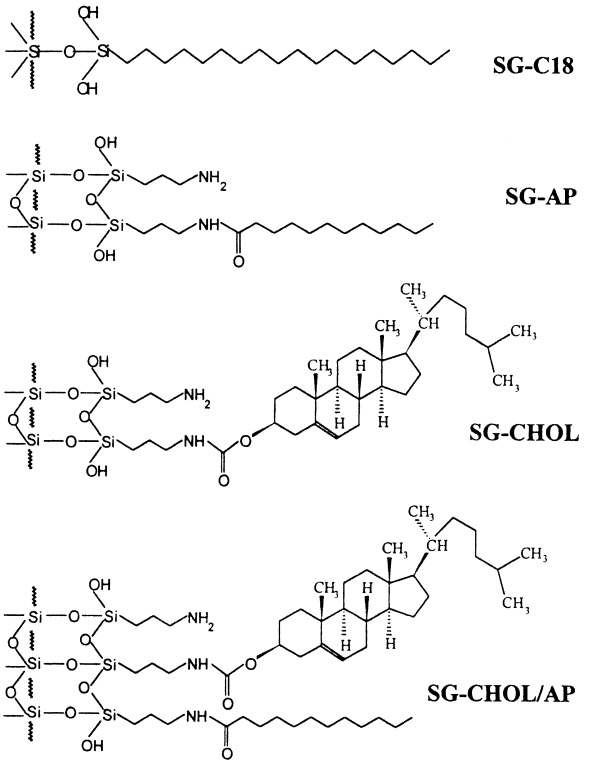


Fig. 2. Chemical structure of stationary phases obtained after modification.

Table 2
The degree of silica coverage with organic ligands based on elemental analysis

Packing	Ist modification stage				IInd modification stage			
	P_C (%)	P_N (%)	α_{RP} (μmol/m^2)	Number of ligands/nm^2	P_C (%)	P_N (%)	α_{RP} (μmol/m^2)	Number of ligands/nm^2
SG-C_{18}	19.02	–	3.74	2.24	–	–	–	–
SG-AP	4.30	1.15	4.60	2.76	15.19	1.20	2.83	1.69
SG-CHOL	4.38	1.25	4.62	2.77	21.19	1.21	2.64	1.58
SG-CHOL/AP	4.47	1.33	4.67	2.80	18.06	1.52	[a]	[a]

[a] The exact coverage density cannot be determined by this technique because two modifiers were used in the second stage of the bonding reaction.

Table 2 presents values characterising the degree of coverage level of the silica support surface represented by coverage density (α_{RP}) corresponding to the concentration of organic phase deposition (μmol) calculated per specific surface area of bare silica (m^2/g) and the number of organic ligands bonded to the surface (nm^2). Both values were calculated on the basis of elemental analysis data using a modified Berendsen equation [28]. By calculation of an α_{RP} value for the phases SG-AP, SG-CHOL and SG-CHOL/AP at the first stage of modification, the coverage density for the intermediate product, which was the SG-NH_2 phase (aminopropyl ligand), was determined. Hence, there are only small differences and the α_{RP} values were reproducible. From the comparison of these three phases it follows that over 55% silanols yielded to conversion (following Unger [4], assuming that on the bare silica surface one finds 8.0 μmol/m^2 silanols which corresponds to ca. 4.0–4.5 OH groups/nm^2). For the SG-C_{18} phase, values of silanols reacting in the synthesis process are ca. 50%, which includes this phase in the interval for packings with a dense coverage [13,29]. For the calculation of α_{RP} values for the second stage of the modification, the matter is more complicated and a determination of these values is possible only for the SG-AP and SG-CHOL phases. A calculation of the α_{RP} value for the SG-CHOL/AP stationary phase seems to be impossible. The established mixed structures of this packing material were confirmed by studies of ^{13}C and ^{29}Si CP/MAS NMR and FTIR. Anyway, it can be concluded that after the first step of modification, phases with controlled and homogenous coverage densities have been achieved. Consequently, after the second stage of modification, in the case of the SG-CHOL/AP phase it can be expected that there is a good shielding of the support surface and a large enough participation of both the cholesteric ligands and the acylamide in the separation process (Table 2).

3.2. Chromatographic characterization and retention mechanism investigation

The present work describes results of application of the new generation of stationary phases, imitating biological membranes, for the separation of estradiols using the RP HPLC system. Both steroids (Fig. 1) possess two functional hydroxyl groups, which determine the properties of these analytes. Substituents in the structure of compounds have a large contribution to the separation process, depending on their nature and on the composition of the binary organic–water mobile phase. The role of substituents in retention is often characterised by the functional group contribution (τ), which is determined by differences in retention of two compounds with various groups (Eq. (1)). This parameter has a specific relationship with the measure of selectivity (α) of the chromatographic system, which becomes evident in comparison of Eqs. (1) and (2). According to Eq. (3) α is one of the factors determining the resolution (R_s) of analytes on the stationary phase. It seems to be obvious that the functional group contribution τ is an indirect indicator of selectivity and as a consequence it should permit the prediction of resolution of compounds on different packings. On the other hand values of τ are related to Hansch substituent constants π (Eq. (4))

$$\tau_x = \log k_{R-X} - \log k_{R-H} = \log (k_{R-X}/k_{R-H}) \quad (1)$$

$$\alpha = k_{R-X}/k_{R-H} \quad (2)$$

$$R_s = 0.25\, N^{1/2} \cdot (\alpha - 1/\alpha) \cdot (k/1+k) \quad (3)$$

$$\pi_x = \log P_{R-X} - \log P_{R-H} = \log (P_{R-X}/P_{R-H}) \quad (4)$$

where k_{R-H}, k_{R-X} are capacity factors for the analyte without substituent and the compound with functional group, α is the selectivity coefficient, N is the number of theoretical plates, P_{R-H}, P_{R-X} are the *n*-octanol–water coefficients for compound without functional group and analyte with substituent.

Comparison of those two constants for different test solutes is one of the methods for predicting retention on stationary phases. To realize this purpose in this study, a series of monosubstituted benzenes were selected and gathered together in Table 3, together with the following parameters: the logarithm of partition coefficients determined for *n*-octanol–water (log P) taken from Refs. [25,30], Hansch constants (π) and the logarithm of retention factor (k) corresponding to a hypothetical pure water eluent (log k_w). This value was calculated by extrapolation of the linear relationship of individual log k data vs. the concentration of methanol in aqueous mobile phases. Middle values of the functional group contribution, obtained on the basis of the capacity factors determined for a binary hydro-organic mobile phase, together with Hansch constants were compared for the chosen analytes on packing materials synthesised in this study. An example of such dependence for the SG-CHOL phase is illustrated in Fig. 3. The remaining data characterising the tested columns are presented in Table 4.

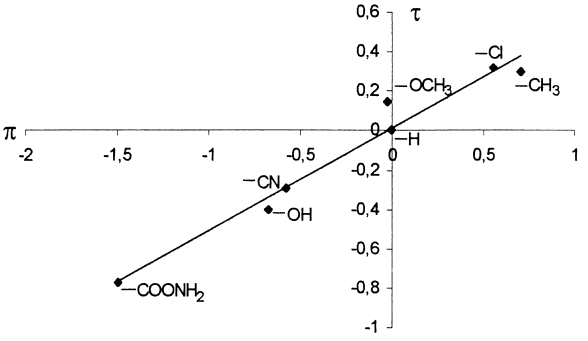


Fig. 3. Relationship between functional group contribution τ and Hansch substituent constants π for a series of monosubstituent benzenes for the SG-CHOL phase.

From the data presented in Table 4 it can be concluded that the highest correlation was obtained for SG-AP ($r^2 = 0.9964$), with the high coverage density reached for this packing and the kind of bonded ligands (possibility of creation of hydrogen-

Table 4
Values of a (slopes), b (intercept) for the linear relationships ($y = ax + b$) and r^2 (regression coefficient) between functional group contribution (τ) and Hansch π constants

Packing	a	b	r^2
SG-C_{18}	0.6429	−0.0917	0.9692
SG-AP	0.4698	−0.0052	0.9964
SG-CHOL	0.5263	0.009	0.9711
SG-CHOL/AP	0.4345	−0.0226	0.9325

Table 3
Structural parameters of test solutes used in the study (for description of log P, π, log k_w, see text)

Compound	Functional group	log P	π	log k_w			
				SG-C_{18}	SG-AP	SG-CHOL	SG-CHOL/AP
Benzene	–H	2.13	0	2.24	1.66	2.21	0.94
Benzoic acid	–C=O(OH)	1.87	–	0.64	0.39	0.64	0.78
Benzamide	–C=O(NH_2)	0.64	−1.49	1.25	0.57	1.14	0.11
Benzonitryle	–CN	1.56	−0.57	1.99	1.32	1.95	0.78
Methoxybenzene	–O–CH_3	2.11	−0.02	2.40	1.74	2.35	0.95
Phenol	–OH	1.47	−0.67	1.52	1.29	1.68	0.53
Toluene	–CH_3	2.73	0.56	2.93	2.20	2.82	1.13
Nitrobenzene	–NO_2	1.85	−0.28	–	–	–	0.90
Chlorobenzene	–Cl	2.89	0.71	3.04	2.38	3.01	–
Bromobenzene	–Br	2.84[a]	0.86	–	–	–	1.80
Iodobenzene	–I	3.30[a]	–	–	–	–	–

[a] Calculated by HyperChem program.

bonds) suggesting the formation of a ''hydrophilic pillow'' (preferential solvatation of residual silanol groups by water molecules) [31]. Consequently, it protects residual silanols from access by the analytes. A slightly lower value of the correlation coefficient for the SG-CHOL materials ($r^2 = 0.9711$) is probably caused by the presence of complex, with respect to volume, and stiff molecules of cholesterol. Also, the presence of additional interaction sites (chiral centers, π-electrons and/or finally the free pair of electrons in the nitrogen atoms in organic ligands) has an influence on the retention of this type of stationary phase. The correlation coefficient for the SG-C_{18} material ($r^2 = 0.9692$), which is theoretically the most hydrophobic, shows that there can be a quite high activity of residual silanols. Based on the data obtained, it should be stressed that both the size and the polarity of the analyte play important roles in the retention mechanism.

Good correlations achieved for the stationary phases studied (Table 4) indicate that the principal mechanism of retention is a partition process, similar to the octanol–water partitioning. The retention on these packings is dependent on hydrophobicity effects. Such a conclusion is in agreement with results of quantitative structure–retention relationships (QSRR) analysis, described earlier in many papers [30]. However, such a conclusion should not exclude the participation of an adsorption mechanism, due to the presence of a population of residual silanols and amino groups. Interactions between phenolic functional groups, localized in the estradiol molecule, and, for example, unreacted amino groups (in the case of SG-AP, SG-CHOL, SG-CHOL/AP) leads to the creation of hydrogen-bonds, which are responsible for the adsorption of the molecules on the surface of the stationary phase. It must be concluded that for the packing materials here, the retention mechanism has a mixed character, connected with the partitioning and the adsorption processes.

3.3. Application

Results from chromatographic investigations of steroids are presented in Fig. 4. The most effective separation of 17-α-estradiol from the diastereoisomer 17-β-estradiol was obtained for the SG-CHOL packing with an acetonitrile–water mixture (60:40, v/v) as a mobile phase in a time not over 11 min. The better resolution for this stationary phase is a consequence of the special properties of cholesterol-based

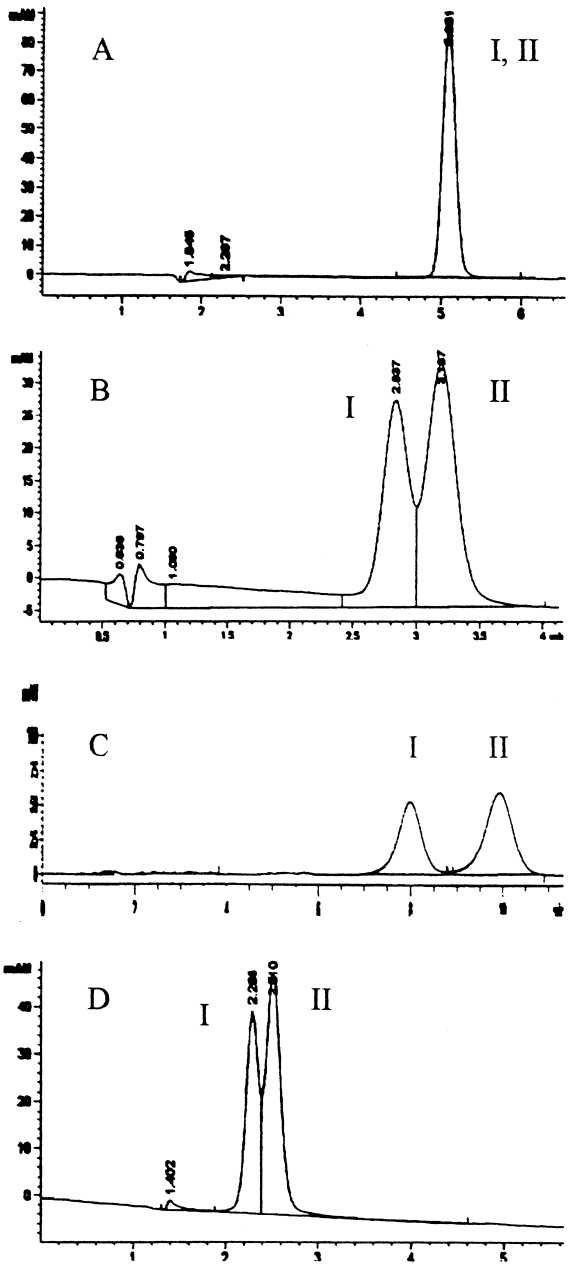


Fig. 4. Separation of 17-α-estradiol (I) and 17-β-estradiol (II) on (A) SG-C_{18}, (B) SG-AP, (C) SG-CHOL, (D) SG-CHOL/AP. Separation conditions: mobile phase, ACN–water (60:40, v/v); flow-rate, 1 ml/min; detection, UV; $\lambda = 254$ nm.

materials (especially participation of chiral centers in the interactions and also orientation of the stationary phase ligands in hydroorganic separation conditions).

The specific nature of the dependence of retention on size and polarity of analyte and ligands appears to be confirmed by the comparison of the dependence of resolution vs. polarity of stationary phase (Fig. 5). Hydrophilic groups localized in the steroid analyzed determine better separation on those stationary phases, where the surface has more polar properties. According to Ref. [24] cholesteric ligands attached to a silica support in SG-CHOL are partially accessible to the test solutes in comparison with for example SG-CHOL/AP material. Also a lower polarity and weaker solvation with solvent molecules is observed for this packing. Results presented in Fig. 5 for the case of the SG-AP material are somewhat surprising, because of the separation of the analytes on the stationary phase, which does not contain any of the cholesteric groups. This is probably a consequence of the presence of the free pair of electrons in the nitrogen atoms of the organic ligands on this packing. This conclusion seems to be confirmed by a comparison of the separation of the estradiol diastereoisomers on the SG-AP and SG-C_{18} stationary phases. For the hydrocarbonaceous material, resolution equals zero, which is in agreement with the hydrophobic character of the bonded organic chains, without specific groups in its structure, such as in the case of the alkylamide stationary phase. On the other hand, although the high correlation coefficient for the SG-AP packing (Table 4) suggests larger possibilities for the prediction of retention on the alkylamide material, the resolution obtained for the separation of estradiols is relatively low. This can be a consequence of the isomeric structure of the analyzed steroid. It should be noted that the results of a comparison of average τ values with Hansch π constants in the case of predicting separation on the studied stationary phases proves to be not sufficient in the analysis of estradiols.

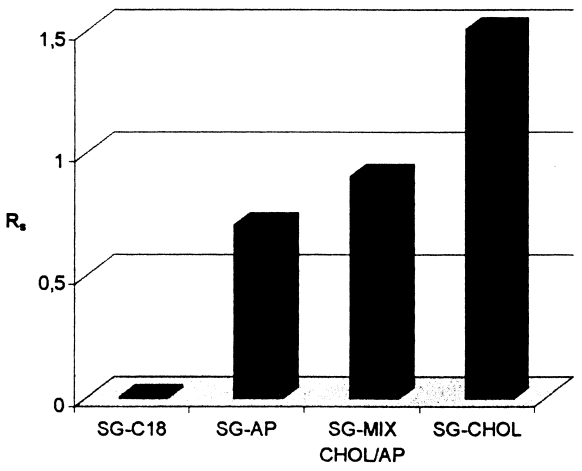


Fig. 5. Comparison of the dependence of resolution vs. polarity of stationary phase for separation of 17-α-estradiol and 17-β-estradiol.

The best resolution of compounds, obtained on the cholesteric material, is caused by the structure of this packing material. Such analysis requires a stationary phase with specific structural properties. Both estradiols are different in the configuration at the chiral center of carbon 17. The molecule of cholesterol possesses eight asymmetric carbons, which makes cholesteric stationary phases a promising material for the separation of enantiomers. Enantioselectivity of stationary phases depends on individual contributions to chiral recognition for different substituents located near a chiral carbon atom (in case of estradiols –OH) [32]. Better shape recognition capabilities for the SG-CHOL material towards the separation of estradiols could be a consequence of liquid crystalline properties of the stationary phase. The expected special properties, caused presumably by the liquid crystal structure of chemically immobilized molecules of cholesterol, were the subject of many previous reports [33–35]. Still it has not been proven that the reason for the higher shape selectivity for these types of stationary phases is their specific configuration in hydro-organic conditions connected with liquid crystal-like behavior.

4. Conclusion

In the chemical modification of bare silica, four stationary phases (SG-C_{18}, SG-AP, SG-CHOL, SG-CHOL/AP) for RP HPLC have been synthesized. The results of the elemental analysis allow classifying them as materials with homogenous and controlled density. Chromatographic measurements of female sexual hormones under the same standard conditions on the synthesized stationary phases with

different polarity properties have been effected. In the present report, new procedures for the separation of estradiol diastereoisomers were developed. It was demonstrated that better resolution for these compounds were obtained for the SG-CHOL material, with an acetonitrile–water (60:40, v/v) mixture as the mobile phase (flow-rate, 1 ml/min; UV detection, $\lambda = 254$ nm), in a time not over 11 min. It can be concluded that the retention mechanism on phases containing immobilised molecules of cholesterol is based on its polarity and ability to recognize specific structures found in isomeric compounds.

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